Minireview

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Angiogenesis

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Regulation of Angiogenesis in Health and Disease

Angiogenesis is a fundamental process by which new blood vessels are formed (1). It is essential in reproduction, development, and wound repair. Under these conditions, angiogenesis is highly regulated, *i.e.* turned on for brief periods (days) and then completely inhibited. However, many diseases are driven by persistent unregulated angiogenesis. In arthritis, new capillary blood vessels invade the joint and destroy cartilage. In diabetes, new capillaries in the retina invade the vitreous, bleed, and cause blindness (2). Ocular neovascularization is the most common cause of blindness and dominates approximately 20 eye diseases. Tumor growth and metastasis are angiogenesis-dependent (3, 4). A tumor must continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow. Furthermore, the new blood vessels embedded in a tumor provide a gateway for tumor cells to enter the circulation and to metastasize to distant sites, such as liver, lung, or bone.

Capillary blood vessels consist of endothelial cells and pericytes. These two cell types carry all of the genetic information to form tubes, branches, and whole capillary networks. Specific angiogenic molecules can initiate this process. Specific inhibitory molecules can stop it. These molecules with opposing functions appear to be continuously acting in concert to maintain a quiescent microvasculature in which endothelial cell turnover is thousands of days. However, the same endothelial cells can undergo rapid proliferation (5-day turnover) during spurts of angiogenesis, for example in wound healing. The proteins which regulate the coagulation system and the family of proteins which regulate the hematopoietic system, including the colony-stimulating factors, interleukins and erythropoietin (5), appear to operate by a similar program. The protein interactions of these latter systems, however, are better understood than are the proteins involved in angiogenesis. Angiogenic factors and inhibitors have been discovered only in the past decade, and while their properties can be listed (Table I), the elucidation of their interactions with each other is only beginning to be uncovered. The same can be said of non-vascular cells, such as macrophages and mast cells, which may modulate the angiogenic response.

Angiogenic Molecules

The first complete purification of an endothelial cell growth factor was based on heparin affinity chromatography (6–8). When the protein was subsequently sequenced (9), it was found to be basic fibroblast growth factor (bFGF).[1] Acidic FGF (aFGF) was also purified by heparin affinity (10, 11). At this writing there are 8 angiogenic polypeptides which have been completely purified, sequenced, and cloned individually in a number of laboratories. These contributions are significant and are presented in Table I. The peptides differ greatly in their biochemical and biological properties. For example, angiogenin, originally isolated from human colon cancer cells, has ribonucleolytic activity (102). Furthermore, the angiogenic peptides also have different target cells. Vascular endothelial growth factor (VEGF, vascular permeability factor) (12–14) and platelet-derived endothelial cell growth factor (15) act mainly as mitogens for vascular endothelial cells. In contrast, the FGFs are pleiotropic. They stimulate the growth of endothelial cells, smooth muscle cells, fibroblasts, and certain epithelial cells. They also promote neurite outgrowth. Under specific conditions they act as differentiating and maintenance factors for nerves (16, 17) while at the same time inhibiting the differentiation of myoblasts.

Several low molecular weight factors are also angiogenic. 1-Butyryl glycerol is secreted by adipocytes that have differentiated from 3T3 fibroblasts (18). The prostaglandins PGE_1 and PGE_2 (19, 21, 22), nicotinamide (23), and related compounds such as adenosine (24, 25) are reported to be angiogenic. Adenosine is a vasodilator that accumulates in response to hypoxia. It is not clear how adenosine induces vasoproliferation. Certain degradation products of hyaluronic acid are angiogenic (26). Recently, (12*R*)-hydroxyeicosatrienoic acid (27) and okadaic acid (28) have been reported to be highly angiogenic.

Several angiogenic factors have been isolated from tumors, wound fluid, and from other tissues, but they have not yet been completely purified or characterized (29, 30). Certain copper complexes are angiogenic. These include copper complexed to the tripeptide Gly-His-Lys (31), ceruloplasmin, and heparin. Furthermore, copper deficiency in rabbits inhibits angiogenesis in the cornea (20) and in brain tumors (32). No satisfactory explanation has been proposed for the role of copper in angiogenesis (33).

Central Questions about the Angiogenic Process

While the angiogenic molecules outlined in Table I are described in detail in several recent reviews (33–40), we wish to focus on the role that these polypeptides play in the angiogenic process. The field of angiogenesis could claim no purified angiogenic molecules before 1983. Now, completely sequenced angiogenic molecules can be tabulated, but we have only a dim conception about how they operate, how they mediate angiogenesis and how they are regulated. Also, most of these molecules have other effects, and the interrelations between the different factors and their effects are still largely unknown. Certain central questions can however, be formulated. They are addressed in this Minireview, and they are also the basis of much new research in this field.

What Is the Role of Heparin and Heparan Sulfate in the Regulation of Angiogenesis?—As we have come to gradually understand the angiogenic process over the past two decades, it is surprising how frequently heparin, heparan sulfate, and their related polysaccharides appear to play a key role. Soon after the heparin affinity of the fibroblast growth factors was discovered (6, 7), it became clear that this property was more than just a useful purification technique. Important physiological functions for this protein-polysaccharide interaction were uncovered. Heparin potentiates the biological activity of aFGF (41), perhaps by increasing the affinity of aFGF for its cell surface receptors (42). Heparin protects aFGF and bFGF from degradation by heat, acid, and proteases (43–45). bFGF is stored in the extracellular matrix and can be mobilized in a biologically active form by heparin or heparan sulfate (46, 47). The binding of FGF to heparan sulfate is a prerequisite for the binding of FGF to its high affinity receptor on the cell surface (48, 49). A specific heparan sulfate proteoglycan has been found to mediate the binding of bFGF to the cell surface (50). Cell-associated heparan sulfate proteoglycans appear to provide a sustained release reservoir that can mediate long-term responses to brief exposures of bFGF (51), and soluble sulfated proteoglycans may permit the diffusion of bFGF in tissues. A specific heparin binding domain has not been clearly

[1] The abbreviations used are: bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; TNF, tumor necrosis factor.

TABLE I

Angiogenic polypeptides

Angiogenic activities of the listed polypeptides were determined by chick embryo chorioallantoic membrane assay, corneal micropocket assay, or hamster cheek pouch assay as described in the references cited in the first column. These assays cannot currently be used to compare potency quantitatively. However, the FGFs and angiogenin appear to be the most potent. VPF, vascular permeability factor; PD-ECGF, platelet-derived endothelial cell growth factor; TGF, transforming growth factor; PDGF, platelet-derived growth factor. +, stimulatory; O, no effect; −, inhibitory.

	M_r	Subunit	pI	Endothelial cell mitogenicity *in vitro*	Other angiogenesis-related biological activitites
			pH		
bFGF (8, 82)	18,000	1	9.6	+	Both bFGF and aFGF (for review see Refs. 33–38) are mitogenic for a wide variety of cell types; bind to heparan sulfate proteoglycan (46, 85) and copper (86); stimulate endothelial cells to migrate and form tubes (82), to increase production of proteases and plasminogen activator (87, 88); and to act as embryonic inducers (89, 90).
aFGF (83, 84)	16,400	1	5	+	
VEGF/VPF (14, 91, 92)	45,000	2	8.5	+	Proliferation activity highly specific for vascular endothelial cells (12, 13); secretory proteins (93–95); increase vascular permeability (96); induce plasminogen activator and plasminogen activator inhibitor in endothelial cells (62); structurally related to PDGF (97–99).
PD-ECGF (15)	45,000	1	5	+	Stimulates endothelial cell DNA synthesis and chemotaxis; proliferation activity not reported; amplifies DNA synthesis activity of FGFs on endothelial cells.[a]
TGF-α (100)	5,500	1	6.8	+	Transforms normal cells into transformed phenotype; binds to EGF receptor (for a review on TGF-α see Ref. 101).
Angiogenin (102)	14,100	1	9.5	O	Stimulates endothelial cells to form diacylglycerol (103) and to secrete prostacyclin (104) by activating phospholipase C and phospholipase A_2, respectively; has a unique ribonucleolytic activity (105, 106) essential for neovascularization.
TGF-β (107)	25,000	2	4	−	Enhances extracellular matrix production; binds to copper[a]; chemotactic for monocytes (for a review on TGF-β see Ref. 108).
TNF-α (109, 110)	55,000	3	4	−	Induces production of bFGF in endothelial cells and enhances its secretion (111); chemotactic for monocytes; activates macrophages (for a review on TNF-α see Ref. 112).

[a] Y. Shing and J. Folkman, unpublished data.

identified in the linear sequence of bFGF (52). Rather, the high affinity heparin binding of bFGF may critically depend upon an intact three-dimensional structure of the growth factor (52). The recently reported three-dimensional structure of bFGF (53) may yield additional insight into its interaction with heparin.

Certain simple sulfated polysaccharides such as β-cyclodextrin tetradecasulfate can substitute for heparin as a regulator of angiogenesis (54). β-Cyclodextrin tetradecasulfate is as good or better than heparin in purifying bFGF by affinity chromatography (55). However, the simplest heparin mimic is sucrose octasulfate (56). Its aluminum salt, sucralfate, is widely used as a treatment of duodenal ulcer. The mechanism of action of this anti-ulcer drug has only recently been discovered; it appears to protect endogenous bFGF that is normally present in the stomach lining from being degraded by gastric acid (57). The angiogenic activity of endogenous bFGF contributes to the healing of peptic ulcers. This finding raises the interesting question of whether protein-polysaccharide complexes in gastrointestinal mucous may regulate growth factor activity in the gut.

How Does a Tumor Cell or a Macrophage Switch to the Angiogenic Phenotype?—During the development of a tumor, there can be a prolonged period (weeks in mice, years in humans) during which the tumor is not angiogenic and is restricted in growth to a few mm^3 (*e.g. in situ* carcinoma). When sufficient cells within the tumor have switched to the angiogenic phenotype, neovascularization may begin. Vascularization is necessary but not sufficient for rapid growth of the primary tumor and for the metastasis of its cells to distant organs.

It is now clear that the angiogenic phenotype may be achieved by more than one mechanism, some of which are diagrammed in Fig. 1. A previously held assumption that tumor angiogenesis could be explained simply by tumor cells releasing angiogenic molecules is no longer tenable. The acquisition of angiogenic activity by tumors appears to be a complex process. Tumor cells do release angiogenic molecules such as VEGF, but it is unclear how this release differs in the pre-angiogenic tumor cell. A more puzzling problem is how bFGF, which lacks a signal peptide, is exported from tumor cells (58, 59). Furthermore, angiogenic activity may be under the control of tumor suppressor genes. Thus, normal cells can secrete a protein such as thrombospondin which inhibits angiogenesis but which is down-regulated during tumorigenesis (60, 61). This concept that tumor cells may produce

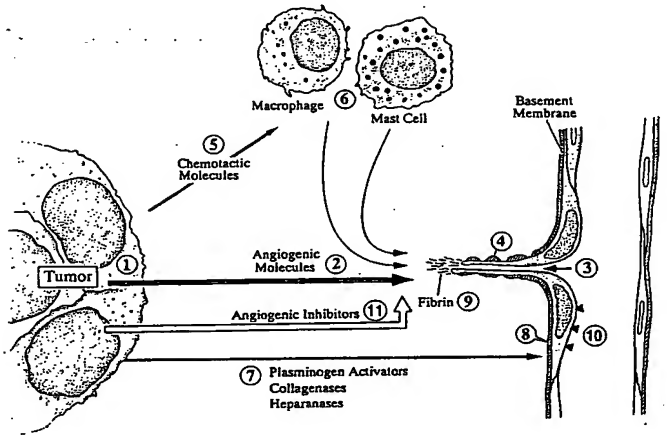

FIG. 1. **Diagram of different mechanisms which may be involved in the switch to the angiogenic phenotype.** These mechanisms can also be targets of antiangiogenic therapy as described in Ref. 40. *1*, some angiogenic molecules are exported out of tumor cells. *2*, angiogenic molecules must diffuse through tissue to reach the vasculature. Heparan sulfate stabilization of bFGF may be an example. *3*, some angiogenic molecules stimulate endothelial cell migration or chemotaxis more potently than DNA synthesis (*i.e.* bFGF). *4*, angiogenic molecules may stimulate production of collagenases and plasminogen activator by endothelial cells, and these proteases may in turn degrade the basement membrane of the parent venule. *5*, tumors may recruit macrophages and mast cells, which themselves can promote tumor angiogenesis. *6*, macrophages may release specific angiogenic molecules or TNF-α, which can recruit more macrophages. *7*, tumor cells may also secrete proteases which contribute to degradation of the basement membrane. *8*, bFGF stored in the extracellular matrix may be mobilized by the factors described in *7*. *9*, VEGF, in addition to being angiogenic, causes increased permeability of the capillary bed. This may lead to leakage of fibrin products into the extracellular space (81). *10*, in some tumors, endothelial cells are found to contain significantly more bFGF than the tumor cells. The mechanism of this phenomenon is unknown. *11*, endogenous inhibitors of endothelial growth secreted by tumors may need to be down-regulated before the release of angiogenic molecules can be sufficiently unopposed so as to induce neovascularization. From Ref. 40 with permission.

factors which either induce or inhibit angiogenesis and that the onset of angiogenic activity is determined by the *balance* of these factors may be generalizable to other tumors. The idea is further supported by recent evidence that tumor cells simultaneously secrete proteases (*e.g.* plasminogen activator) and their inhibitors

(*e.g.* plasminogen activator inhibitor) and that the balance between them precisely regulates the level of extracellular proteolysis and thus promotes or suppresses angiogenesis (62, 63).

Tumor cells may not be the sole source of angiogenic molecules within a tumor. Tumors may recruit macrophages and then activate them to secrete angiogenic activity (64). Mast cells may also be recruited by tumors. Loaded with heparin, mast cells may amplify the effect of bFGF produced by tumor cells. Even the endothelial cells that live in a tumor as part of its neovasculature may increase their own production of bFGF (65). In addition to the angiogenic factors exported by tumor cells and the host cells within the tumor, bFGF is also stored in the extracellular matrix. This bFGF may be mobilized by collagenases or heparinases secreted either by tumor cells or by associated non-neoplastic cells.

Another puzzle is that when tumor angiogenesis is mediated by bFGF, histological sections reveal that vascular endothelial cell proliferation dominates, but smooth muscle cell and fibroblast proliferation are less apparent. Even in an experimental system where tumor cells are transfected with bFGF containing a signal sequence and the bFGF is highly secreted, the resulting tumor stimulates predominantly vascular endothelial cells (66).

The onset of wound healing angiogenesis may be less complex than tumor angiogenesis. Macrophages are the major source of angiogenic activity in a healing wound. Macrophages release bFGF as well as TNF-α. Macrophage angiogenic activity is switched on in the depths of a wound when the oxygen tension is low and lactate is high from anaerobic metabolism (67). Macrophage angiogenic activity is also increased during phagocytosis of fibrin or another particle. The hypoxic center of a tumor is a good place for macrophages to become angiogenic. The concept of a tumor as a non-healing wound is based in part on this observation (68).

What Conditions in the Microvasculature Are Required for Initiation of Neovascularization?—The presence of tumor cells or macrophages that have switched to the angiogenic phenotype may be necessary but not sufficient for neovascularization to be initiated. A number of barriers in the microvasculature may have to be overcome. Resting endothelial cells are guarded by specialized cells, pericytes (69), which help to maintain endothelium in a quiescent, non-proliferating state (70). Pericytes in the microvasculature appear to inhibit endothelial proliferation. This inhibition requires cell-cell contact between pericytes and endothelial cells which leads to the activation of latent transforming growth factor-β produced by both cells. It is not known how tumors overcome this barrier.

Endothelial cells *in vitro* are refractory to growth factors when they are confluent (*e.g.* foreshortened) (71). Endothelial cell spreading or elongation increases sensitivity to specific growth factors, for example bFGF (72). It is unclear whether this phenomenon is operating *in vivo*. However, the small venules from which new capillary sprouts arise under stimulation of an angiogenic molecule (*i.e.* bFGF) appear histologically to contain crowded endothelial cells analogous to a confluent cell culture. How then does angiogenesis start? Vasodilation of the parent venule is an early event in angiogenesis that occurs before the emergence of the first capillary sprout. This may stretch endothelial cells so that they become responsive to bFGF or other growth factors. Vasodilation has been overlooked as an important initial step in the cascade of events necessary to build a whole capillary network. The mechanism of this vasodilation is not known nor is it clear whether bFGF itself is responsible.

During angiogenesis, endothelial cells change their morphology from tubular (parent venule or capillary) to flat and elongated (growth of sprout) and back to tubular (established capillary blood vessel). These changes can be recapitulated *in vitro* (73). Bischoff (74)[2] has recently identified a novel protein produced by endothelial cells which appears to "seal" the tube. Synthesis and degradation of this protein may play a role in the morphological changes which endothelial cells undergo during angiogenesis.

Why Are Angiogenic Factors Categorized as Direct and Indirect?—A longstanding problem in angiogenesis research is how to determine the mechanism of action of an angiogenic molecule. This usually requires that the target cell be determined for a given angiogenic factor. Target cell specificity, however, is usually determined *in vitro*, while angiogenic activity is analyzed *in vivo*. When the two methods correlate, the angiogenic factor is called "direct" because it stimulates endothelial cell proliferation or migration directly. Examples are aFGF, bFGF, transforming growth factor-α, and VEGF. When an angiogenic factor fails to stimulate endothelial cells *in vitro* it is called "indirect," because it is assumed that the endothelial proliferation and/or migration which is observed *in vivo* must have been induced by some other factor or cell (*e.g.* a macrophage), perhaps indirectly mobilized by the original angiogenic molecule. Examples are TNF-α and angiogenin. The usefulness of this classification is limited for the obvious reason that cultured endothelial cells are not the same as the endothelial cells which are proliferating during the angiogenic reaction *in vivo*. Furthermore, even for a "direct" angiogenic factor, it is difficult to rule out the possibility of intermediate factors as the proximate stimulus of angiogenesis.

Another problem is that both direct and indirect angiogenic molecules appear to have paradoxical activities *in vivo* depending upon *route* of administration. Thus, TNF-α is angiogenic when injected *extra*vascularly but may cause tumor necrosis in some animals when it is injected *intra*vascularly. One explanation is that in the extravascular position, TNF-α is chemotactic for macrophages and they actually induce the angiogenesis, while intravascular TNF-α may induce microvascular coagulation, especially in tumor vessels (75). bFGF is angiogenic when injected extravascularly, but intravascular infusion does not stimulate endothelial DNA synthesis (76) unless capillary growth is already under way (77). The mechanisms of these contrasting endothelial responses are unknown. It is possible that vascular endothelial cells may respond differently to growth factors or to inhibitors depending on whether the factor is exposed to the luminal or abluminal surface of the cell.

Clinical Applications

Certain applications of angiogenesis research are now in clinical trial. These fall into three areas: diagnostic applications, acceleration of angiogenesis in wound healing, and inhibition of angiogenesis in neoplasia. Quantitation of angiogenesis in biopsy specimens of breast cancer provides an independent marker of future metastatic risk (78). Topical application of bFGF in chronic wounds accelerates angiogenesis and wound healing (79). Life-threatening hemangiomas can be successfully treated by utilizing the antiangiogenic property of α-interferon (80).

Other applications are being prepared for clinical trial. The quantitation of elevated angiogenic factors in the blood and urine of cancer patients is being evaluated to determine prognosis and to guide therapy. Orally delivered bFGF which accelerates angiogenesis and healing in duodenal ulcers (57) is being studied for future clinical trial in patients with refractory gastrointestinal ulceration. A potent fungal-derived angiogenesis inhibitor is under study for eventual use as an anti-cancer agent (113).

Continued advances in understanding the mechanism of the angiogenic process at the biochemical and molecular levels may provide further diagnostic and therapeutic benefits in a variety of diseases.

[2] J. Bischoff *et al.*, unpublished data.

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